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# *In vitro* Cytotoxicity of *Gmelina arborea roxb* (Gambhari) on HL-60 Cell Lines

[1]Rohit Sahu, [2]Goli Divakar, [2]Kalyani Divakar and [1]Paras Sharma
[1]BVM College of Pharmacy, Gwalior, Madhya Pradesh, India
[2]Acharya and B.M. Reddy College of Pharmacy, Bangalore, Karnataka, India

*Corresponding Author: Rohit Sahu, Department of Pharmacology, BVM College of Pharmacy, Shivpuri Link Road, Gwalior, Madhya Pradesh, India Tel: +91 9691208279, 8989923987*

## ABSTRACT

*Gmelina arborea roxb* commonly known as 'Gambhari' tree, the various parts of the plants are widely used in diarrhoea, anti-pyretic, thirst, anemia, leprosy, ulcers, consumption, strangury and vaginal discharges. We tested the cytotoxic potential of *Gmelina arborea* roxb in HL-60 cells. Aqueous Extract of *Gmelina arborea* roxb (AEGA) was tested at the various concentrations 5, 10, 15 and 20 mg in MTT assay, Cell Viability assays and clonogenic assay. Our study shows that AEGA inhibits cell growth and decrease the cell viability. The AEGA inhibits cell proliferation at a dose and time dependent manners measured by MTT assay. The AEGA very significantly decreased the cell viability of HL-60 Cells after 24 and 48 h compared to the control cells. In the semisolid culture, the number of colonies decreased significantly ($p<0.01$) in a dose-dependent manner. Overall, AEGA has shown a substantial and significant anti cancer activity in all the models. This protective effect might have been mediated by apoptosis mechanisms.

**Key words:** MTT assay, cell viability assays, clonogenic assay, cytotoxicity, AEGA, HL-60

## INTRODUCTION

Cancer is a disease characterized by uncontrolled proliferation of the cell that have transformed from the normal cell of the body (Gupta, 2004). Cancer arises as a result of genetic and epigenetic changes, inactivation of tumor suppressor genes and the activation of oncogenes. It is one of the major causes of death in the developed nations (Rang *et al*., 2005). The MTT assay is a laboratory test and a standard quantitative colorimetric assay (an assay which measures changes in color) for measuring mammalian cellular growth, cell survival and cell proliferation based on the ability of live cells. It can also be used to determine cytotoxic potential of medicinal agents and other toxic materials. Cell viability assays measure the percentage of a cell suspension that is viable. Loss of cell viability is most often measured as loss of membrane integrity. This event may be primarily due to necrosis or secondarily to apoptosis. This is generally accomplished by trypan blue exclusion technique (Freshney, 2005). A clonogenic assay is a microbiological technique for studying the effectiveness of specific agents on the survival and proliferation of cells. *Gmelina arborea* roxb Verbenaceae Family, an important commercial timber species has been used in Ayurveda, Since ancient times. The medicinal parts of the plant are the leaves, bark, root and fruit which had numerous uses in traditional medicine. Its decoction is used as a diuretic, appetite stimulant and in the treatment of various stomach disorders, fevers, skin problems, liver disorders. *In vitro* studies showed that it possess antioxidant activity, anti-inflammatory activity, anti diarrhoeal

activity and wound healing activity (Hosny and Rosazza, 1998; Agrawal *et al*., 1994; Agunu *et al*., 2005; Shirwaikar *et al*., 2003; Sinha *et al*., 2006; Satyanarayana *et al*., 2007).

Therefore, investigation into the traditionally used medicinal plants is valuable as a source for potential chemotherapeutic drugs and as a safety measure for the continued use of medicinal plants. Thus, the aim of this preliminary study was to evaluate the cytotoxic effect induced by *Gmelina arborea,* when HL-60 cells were exposed to various concentrations of AEGA.

## MATERIALS AND METHOD

**Plant material:** Plant material used in this study was *Gmelina arborea* roxb. The leaves of *Gmelina arborea roxb* were shade dried and reduced to coarse powder in a mechanical grinder. The powdered material obtained was then subjected to extraction using Distill water in a Soxhlet extractor. The extract obtained was evaporated at 100°C to get a semisolid mass. The extract prepared at the Department of Pharmacology, Acharya and B.M. Reddy College of Pharmacy, Bangalore, India. The stock solution of plant extract (AEGA) 100 mg $mL^{-1}$ was Prepared with sterile water and filtered through syringe filter (0.45 μL). Cells were exposed to various concentrations 5, 10, 15 and 20 mg of AEGA.

**Chemicals and reagents:** Agar (Himedia), DMSO (Karnataka Fine Chemicals, Bangalore), Fetal bovine serum (GibcoBRL, USA), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Lobbe Chemicals), RPMI-1640 medium (sigma) and trypan blue (Karnataka Fine Chemicals, Bangalore). All chemicals used in this study were of analytical grade purity and all test solutions were freshly prepared before each experiment.

**Cancer cell and cell culture:** The HL-60 cancer cells were used for the anti cancer assays. These cancer cells were obtained from the National Center for Cell Science (NCCS, Pune, India) and were maintained at Institute of Animal Health and Veterinary Biologicals, Hebbal, Bangalore. The cells were maintained in their logarithmic phase of growth in RPMI 1640 medium (Sigma), supplemented with heat-inactivated 7% fetal bovine serum (Gibco), in humidified air with 5% $CO_2$.

**Evaluation of cell proliferation by MTT assay:** The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) assay is a common method used to assess cell proliferation and cytotoxicity.

**Procedure:** About $1\times10^4$ exponentially growing cells were seeded per well in 96 well plates. Cells were exposed to various concentrations 5, 10, 15 and 20 mg of AEGA. Incubated the plate for 24 h at 37°C in 5% $CO_2$/95% humidified air. Centrifuged the plate at 2000 rpm for 10 min and discarded the supernatant. Added 100 μL of MTT (2 mg $mL^{-1}$) into each well of 96 well plates and incubated at 37°C in 5% $CO_2$/95% humidified air for 4 h. Centrifuged the plate at 2000 rpm for 10 min and discarded the supernatant. The precipitated formazan salt was dissolved in 100 μL of DMSO. The plate samples were read at 492 nm with an ELISA micro plate reader (Sharma *et al*., 2007; Lantto *et al*., 2009). Each experiment was performed in triplicate. The percentage cell viability was calculated using equation:

$$\text{Cell viability}\,(\%) = \frac{\text{Absorbance of treated cells}}{\text{MTT reduction}(\%)\ \text{Absorbance of untreated cells}} \times 100$$

**Cell viability assay:** Cell Viability assay measures the percentage of a cell suspension that is viable. Loss of cell viability is most often measured as loss of membrane integrity. This event may be due to primarily necrosis or secondarily to apoptosis. This is generally accomplished by trypan blue exclusion technique, where cells with an intact membrane are able to exclude the trypan blue while cells without an intact membrane take up the trypan blue due to alteration in the membrane permeability.

**Procedure:** About $3\times10^4$ exponentially growing cells were seeded per well in 24 well plates. Cells were exposed to various concentrations 5, 10, 15 and 20 mg of AEGA. Incubated the plate at 37°C in 5% $CO_2$/95% humidified air. After 24 and 48 h of incubation the cell culture were taken in tube and centrifuged them at 2000 rpm for 10 min and discarded the supernatant. Resuspended the cells pellets in 1 mL of growth medium. Numbers of cultured cells in the different wells were counted using a hemocytometer after staining with 0.4% trypan blue (Sharma *et al*., 2007; Jimenez *et al*., 2008; Yang *et al*., 2000). Each experiment was performed in triplicate. The percentage cell viability was calculated using equation:

$$\text{Cell viability}\,(\%) = \frac{\text{No. of viable cells}}{\text{Total No. of cells (viable+dead)}} \times 100$$

**Clonogenic assay:** A clonogenic assay is a microbiological technique for studying the effectiveness of specific agents on the survival and proliferation of cells. It is frequently used in cancer research laboratories to determine the effect of drugs or radiation on proliferating tumor cells.

**Procedure:** In 24 well plates, 2% of agar was added as bottom layer and allowed for solidification. After solidification, HL-60 cells ($5\times10^5$ per sample) were cultured with different concentration 5, 10, 15 and 20 mg $mL^{-1}$ of AEGA, 1 mL RPMI-1640, supplemented with 0.3% agar and 20% fetal bovine serum were added to the above bottom layer and incubated at 37°C in a fully humidified atmosphere containing 10% $CO_2$ in air (NuAire, Inc, MN). Each experiment was performed in triplicate. Colonies (greater than 40 cells) were scored after 10 days by using inverted microscope at 40x. The survival of clonogenic cells was expressed as ratio between the mean number per plate of colonies surviving on triplicate plates (Saito *et al*., 1986; Ellwart *et al*., 1988; Santos *et al*., 2006).

**Statistical analysis:** All the Values are expressed as mean±SEM in triplicate. Statistical significance was set at $p<0.05$ compared with control group. Data were analyzed using ANOVA followed by Dunnet comparison test to observe any significant differences amongst the dosage sets and harvest periods to observe the cytotoxicity effects. All the statistical analysis was performed using INSTAT statistical program.

## RESULTS

**Cell proliferation by MTT assay:** The HL-60 cells were treated for 24 h with different concentrations of AEGA. The cell viability was estimated using the MTT assay, which measures the metabolic activity of mitochondria. As shown in Table 1, all the used concentrations of AEGA decreased the production of formazan compared to the untreated control cells. With all concentrations (5, 10, 15 and 20 mg $mL^{-1}$) of AEGA, cell viability was decreased very significantly

Table 1: Effect of AEGA on proliferation of HL-60 cells (MTT assay)

| AEGA (mg mL$^{-1}$) | Absorbance at 492 nm | Cell viability (%) (MTT reduction) |
|---|---|---|
| Control | 1.3120±0.062 | 100.00±0.11 |
| 5 | 1.2780±0.211$^{ns}$ | 97.40±0.17** |
| 10 | 0.8610±0.030* | 65.60±0.05** |
| 15 | 0.0830±0.006** | 6.30±0.11** |
| 20 | 0.1510±0.013** | 11.50±0.23** |

Values are expressed as Means±SEM in triplicate. **p<0.01, *p<0.05, ns: p>0.05 AEGA: Aqueous extract of *Gmelina arborea* roxb

Table 2: Effect of AEGA on HL-60 cell growth (Trypan blue exclusion assay)

| | 24 (h) | | | 4 8 (h) | | |
|---|---|---|---|---|---|---|
| AEGA (mg mL$^{-1}$) | Total no. of cells | Total no. of viable cells | Cell viability (%) | Total no. of cells | Total no. of viable cells | Cell viability (%) |
| Control | 130±2.88 | 122±2.30 | 93.83±0.31 | 320±6.92 | 304±3.46 | 95.00±0.98 |
| 5 | 168±0.57** | 144±2.30** | 85.66±1.06** | 340±9.23$^{ns}$ | 280±6.92** | 82.33±0.20** |
| 10 | 152±3.46** | 112±1.15* | 73.66±0.92** | 340±4.61$^{ns}$ | 240±1.73** | 70.53±0.43** |
| 15 | 170±5.77** | 40±2.30** | 22.83±1.14** | 380±1.15** | 120±2.30** | 19.44±0.51** |
| 20 | 170±1.73** | 80±2.88** | 47.00±1.21** | 343±1.73$^{ns}$ | 143±1.73** | 41.60±0.28** |

Values are expressed as Means±SEM in triplicate: **p<0.01: *<0.05, ns: p>0.05 compared with control group, AEGA: Aqueous extract of *Gmelina arborea* roxb

Table 3: Effect of AEGA on colony formation of HL-60 cells (clonogenic assay)

| AEGA (mg mL$^{-1}$) | No. of colonies |
|---|---|
| Control | 159±8.93 |
| 5 | 140±11.54$^{ns}$ |
| 10 | 860±13.85** |
| 15 | 420±4.61** |
| 20 | 290±5.19** |

Values are Means±SEM in triplicate cultures. **p<0.01, ns: p>0.05, compared with the control group, AEGA: Aqueous extract of *Gmelina arborea* roxb

compared to the control cells. The MTT reduction (% of control values) data for the AEGA in HL-60 cells after 24 h were 97.4, 65.6, 6.3 and 11.5% at the concentration of 5, 10, 15 and 20 mg mL$^{-1}$, respectively.

**Cell viability assay:** As shown in Table 2, all the used concentrations of AEGA (5, 10, 15 and 20 mg mL$^{-1}$) showed concentration and time dependent effect. The cell viability was decreased very significantly (p<0.01) compared to the control cells. The % cell viability data for the AEGA in HL-60 cells after 24 h were 85.66, 73.66, 22.83 and 47.00% at the concentration of 5, 10, 15 and 20 mg mL$^{-1}$ and after 48 h were 82.33, 70.53, 19.44 and 41.60% cell viability at the concentration of 5, 10, 15 and 20 mg mL$^{-1}$, respectively.

**Clonogenic assay:** In the semisolid culture, the number of colonies decreased significantly (p<0.01) in a dose-dependent manner in experimental groups. Table 3 showed the effect of AEGA on colony formation of HL-60 cells. After incubation for 10 days, colonies with more than 40 cells were counted under an inverted microscope. The no of colonies were 140, 86, 42 and 29 at 5, 10, 15 and 20 mg mL$^{-1}$ doses, respectively.

## DISCUSSION

The effect of AEGA on the growth of HL-60 cancer cells was determined by using the MTT assay. The MTT assay quantifies metabolically viable cells by their ability to reduce MTT. The advantages of the MTT assay include rapid semi-automated reading, objective assessment, comparative low cost, high reproducibility, low number of cells required and the facility to quantify cells grown in suspension, on monolayer or in spheroids or colonies (Price and McMillan, 1990).

When the HL-60 cells were treated for 24 h with different concentrations of AEGA, the cell viability was estimated using the MTT assay, which measures the metabolic activity of mitochondria. As shown in Table 1, all the used concentrations of AEGA, very significantly decreased the percentage cell viability.

The assay is based on the selective reduction of the tetrazolium salt, MTT, by cells which remain viable after exposure and incubation with antitumor effector cells. Mitochondrial dehydrogenases at the sites of cytochromes b and c in viable cells convert the yellow form of the salt to an insoluble, intracellular purple formazan. Solubilized formazan can then be quantitated spectrophotometrically and the results related to the proportion of viable tumor cells in a population incubated with antitumor effectors (Heo *et al*., 1990).

Some phenolic compounds increased the dehydrogenase activity. It is also reported that low concentrations of some phenolic compounds stimulated the proliferation of human pulpal fibroblasts (Kasugai *et al*., 1991).

A reduction in cell growth and induction in cell death are two major ways to inhibit tumor growth (Sharma *et al*., 2007). Reduction in cell growth or induction in cell death may be due to presence of phenolic compounds in AEGA. The exact mechanism of action of AEGA is still unclear. The effect of AEGA on the proliferation of HL-60 cancer cells was determined by using the trypan blue dye exclusion method.

As shown in Table 2, all the used concentrations of AEGA showed concentration- and time dependent effect. The cell viability was found to decrease very significantly ($p<0.01$) in comparison to the control cells.

The Trypan blue dye enables visual distinction of viable and non-viable cells since it will stain those with damaged membrane, possibly preceding death. Cell number reduction may occur not only by a cytostatic effect, as in unpaired DNA replication but also by a cytotoxic effect, like death induction (Jimenez *et al*., 2008). The exact mechanism of action of AEGA is still unclear.

Tannins are plant secondary metabolites that are widely distributed in the plant kingdom. The antitumor activities of a series of naturally occurring tannins have been demonstrated on several tumor cell lines, such as malignant melanoma (RPMI-7951), lung carcinoma (A-549), ileocecal adenocarcinoma (HCT-8), epidermal carcinoma of nasopharnyx (KB) and medulloblastoma (TE-671) tumor cells (Yang *et al*., 2000).

AEGA contained tannins and the anti cancer activity may be due to presence of tannins. The cytotoxic effect of AEGA might have been mediated by apoptosis in HL-60 cells.

The ability of human tumor cells to undergo divisional growth in a semisolid matrix has been demonstrated by a number of investigators (Page *et al*., 1988). Cell proliferation was significantly inhibited by AEGA, examined by trypan blue dye exclusion method (Table 2) and MTT assay (Table 1). In the semisolid culture, the number of colonies decreased very significantly ($p<0.01$) in a dose-dependent manner in experimental groups (Table 3).

AEGA effectively reduced the malignancy and suppressed the regeneration potential of tumor cells. Probably the HL-60 cell line was highly sensitive to the anti-proliferation effect of AEGA.

A wide variety of anti-cancer drugs exhibit cytotoxic effect by interfering with cell-cycle kinetics. These drugs are effective against cells that are proliferating and produce cytotoxic effect either by damaging the DNA during the S-phase of the cell cycle or by blocking the formation of the mitotic spindle in M-phase. Anti-tumor drugs that interact with microtubules and tubulin are known to block mitosis and induce cell death by apoptosis. Most of the plant derived anti-cancer drugs affect the microtubule dynamics of the cell and induce persistent modification of biological processes and signaling pathways that ultimately lead to apoptotic death (Sehgal *et al*., 2006).

Overall, AEGA has shown a substantial and significant anti cancer activity in all the models. This protective effect might have been mediated by apoptosis mechanisms. Therefore, further studies related to the mechanisms of apoptosis, e.g., caspase activation and gene expression are needed to understand the effects of AEGA on the cell viability and cell death in detail. Moreover, further insight into the precise mechanism of action is essential to exploit the complete potency of AEGA and increase its usage in contemporary medicine.

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